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(54) Title: FLUOROGENIC PROTEASE SUBSTRATES DOUBLY LABELLED WITH RHODAMINE MOIETIES

(57) Abstract: Fluorogenic protease substrates are provided that comprise a peptide doubly labelled via thiol groups of the peptide with an alkyleneamidotetramethylrhodamine (alkyleneamido-TMR) group. Preferred substrates are doubly labelled with substantially pure 5-methyleneamido-TMR or 6-methyleneamido-TMR. Methods of preparing the substrates are provided, which comprise reacting the unlabelled peptide with haloalkylamido-TMR (preferably iodoacetamido-TMR). More generally, fluorogenic protease substrates are also provided which comprise a peptide doubly labelled with the same rhodamine derivative, where the two labels, and their linkages to the peptide, are substantially isomerically identical. Also provided are related methods for assaying protease activity in a sample, kits for use in such methods, and solid supports bearing the substrates of the invention. Self quenching of fluorescence occurs in the multi labelled protease substrate due to dye stacking. Cleavage of the peptide by proteases leads to an increase in fluorescence intensity.

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INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/445 C12Q1/37

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07K C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 01 18238 A (PACKARD BEVERLY S ;KOMORIYA AKIRA (US); ONCOIMMUNIN INC (US)) 15 March 2001 (2001-03-15)	1-17, 24-32
Y	paragraph (V.C) on pages 37-39; paragraphs (VI.B) and (VI.C) on pages 41 and 42; paragraph (X) on pages 51-52; claims 1,7,8,16-20; examples 2,5,7; tables 11,12 ----- -/--	18-20



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

*** Special categories of cited documents :*****A*** document defining the general state of the art which is not considered to be of particular relevance***E*** earlier document but published on or after the international filing date***L*** document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)***O*** document referring to an oral disclosure, use, exhibition or other means***P*** document published prior to the international filing date but later than the priority date claimed***T*** later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention***X*** document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone***Y*** document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.***&*** document member of the same patent family

Date of the actual completion of the international search

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15.07.03

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INTERNATIONAL SEARCH REPORT

Inter Application No
PCT/GB 03/00045

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GRAMA LASZLO ET AL: "Global configuration of single titin molecules observed through chain-associated rhodamine dimers." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES, vol. 98, no. 25, 4 December 2001 (2001-12-04), pages 14362-14367, XP002239037 http://www.pnas.org December 4, 2001 ISSN: 0027-8424	1-3,8, 14,16, 18-20,31
Y	abstract, page 14362, right-hand column, lines 14-19,35-39; page 14366, left-hand column, lines 10-16	18-20
X	--- GEOGHEGAN K F ET AL: "Dye-pair reporter systems for protein-peptide molecular interactions." BIOCONJUGATE CHEMISTRY. UNITED STATES 2000 JAN-FEB, vol. 11, no. 1, January 2000 (2000-01), pages 71-77, XP002239038 ISSN: 1043-1802 cited in the application abstract, figure 1, page 72, left-hand column, 2nd and 3rd paragraphs	1,2,10, 13-15,17
P,X	--- BLACKMAN, MICHAEL J. ET AL: "Structural and Biochemical Characterization of a Fluorogenic Rhodamine-Labeled Malarial Protease Substrate" BIOCHEMISTRY (2002), 41(40), 12244-12252, XP002239039 page 12245, paragraph joining left- and right-hand columns; page 12249, right-hand column, 2nd paragraph; figures 1,3,8	1-20, 24-32
A	--- WO 98 50579 A (MINNESOTA MINING & MFG) 12 November 1998 (1998-11-12) abstract; claims 1,11; example 1 -----	1-20, 24-32

INTERNATIONAL SEARCH REPORT

international application No.
PCT/GB 03/00045

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-20, 24-32(partially)

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. Application No

PCT/GB 03/00045

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0118238 A	15-03-2001	AU 7368800 A CA 2384021 A EP 1214445 A JP 2003508080 T	10-04-2001 15-03-2001 19-06-2002 04-03-2003
WO 9850579 A	12-11-1998	AU 4355097 A CN 1254383 A EP 0980440 A JP 2002506343 T	27-11-1998 24-05-2000 23-02-2000 26-02-2002